Supplementary: Protocols

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Microplate Reading

To determine the optimal lactate sensor to integrate in our RIOT system, we designed nine constructs that are expected to express Alanine Racemase (ALR) in the presence of Lactate. The expression of ALR will rescue D-Alanine auxotrophic mutant bacteria grown in media without D-Alanine supplement. More information about the design for these RIOT sensors can be found in our Part Collection

Procedure

1. Measure OD of overnight cultures after 10x dilution: add 900 µl of LB into a cuvette and blank, then add 1 µl of overnight culture, mix and take measurement. Record the corrected OD
2. Prepare 5 ml of M9 (with glucose added) in a 15 ml tube for each constructs
3. Calculate the volume of stock culture need to be added in to 5 ml of M9 to obtain the starting OD of 0.1
4. Aliquot the required volume of stock culture and centrifuge at 6000 rpm for 1 min. Resuspend with M9.
5. Add 2 µl of D-Alanine (50 mg/ml) into 3 wells of a 96-well plate for each constructs as a triplicate.
6. Add 2 µl of each lactate concentration, **1 M**, **10^{-1} M** and **10^{-2} M** into 3 wells for each constructs. The final working lactate concentrations will be **10^{-2} M**, **10^{-3} M** and **10^{-4} M**.
7. Dispense 200 µl of each constructs into each well with triplicate.
8. Dispense 200 µl of M9 into 3 wells and 200 µl of water into 3 wells for blanking.
9. Place the 96-well plate into the plate reader for 6-8 hours at 37 degree.
10. Measure OD600 at 15 minutes interval.

GFP Microscopy Assay Protocol

1. Set up an overnight culture of bacteria in a culture tube, with 5mL of LB, plus the appropriate concentration and type of antibiotic. Incubate in a shaking incubator overnight at 37^{o}C.
2. Prepare the following lactate concentrations: 0 M, 10^{-3} M and 10^{-2} M in respectively labelled 1.5µl microcentrifuge tubes. Each tube should have approximately 500µl of content. Store the three tubes in the -20^{o}C until time to use.
3. Prepare a 25mL media as follows: 25mL M9 media with 5µg/mL of D-alanine, and the appropriate antibiotic concentration and type. Store in the 4^{o}C until time to use.
4. On the next day, collect the culture tube and centrifuge at 5000 rpm for 1 minute.
5. Discard the LB media. Resuspend the cell pellet in 5mL of prepared M9 media.
6. Using the spectrophotometer, measure the OD600 of the resuspended bacteria culture. Add 900µl of LB into a cuvette as blank. Add 500µl of LB into another cuvette and add 500µl of the resuspended culture. Multiply the obtained OD600 measurement by a factor of two (2) to account for the 1x dilution. This will give $OD600_1$. Calculate the volume of bacterial culture to be added (V_f) and the volume of prepared M9 media (1000µL – V_f) to be added to obtain 1mL bacteria sample of $OD600_f = 0.400$.

$$OD600_1V_1 = OD600_fV_f$$

7. Add the calculated volumes into a labelled 1.5µl microcentrifuge tube and incubate in a shaking incubator at 37^{o}C for approximately 30 minutes.
8. Thaw the three lactate tubes and place on ice. Collect cell supernatant of Hela and HepG2 from the -80^{o}C freezer, thaw, and place on ice. Afterwards, collect the diluted bacteria sample.
9. Set up five fresh and labelled 1.5µl microcentrifuge tubes. To each, add 20µl of each lactate concentration (0 M, 10^{-3} M, 10^{-2} M) and 180µl of the diluted bacteria sample to make up 200µl of reaction mix. To the remaining two tubes, add 40µl of each cell

supernatant (Hela and HepG2) and 160µl of the diluted bacteria sample to make 200µl of reaction mix, with 5x dilution of the samples.

10. Incubate in a shaking incubator at 37^{o}C for 3 to 4 hours.
11. Set up the microscope and NIS-Elements Viewer for GFP assay at 100x (with oil immersion) and set to bright-field at the appropriate light intensity, along with labelling of the polylysine slides. Afterwards, collect the tubes.
12. Add 5µl of sample (with the highest lactate concentration, 10^{-2} M) onto the appropriately labelled slide and cover with the slip. Do not move the slide for approximately 3 minutes. Invert the slide and attempt focus for examination under the microscope.
13. Capture approximately 50 bacteria with GFP view and BF view for later analysis.
14. Continue with other lactate concentrations in the order: lactate 0 M, lactate 10^{-3} M, Hela, then HepG2.

Mammalian cell supernatant preparation

Materials

- Cell lines: HeLa and HepG2

- Media: DMEM with phenol red

DMEM without phenol red

Fetal Bovine Serum

Penicillin/Streptomycin

Trypsin

1X PBS

Procedures

1. Revive cells from frozen vials and subculturing 80% confluent as in Cell culture protocol
2. Enumerate cell density by a hemocytometer. Seed 10^6 cells into each well.
3. Resuspend with phenol free DMEM and incubate at 37ºC for 6 hours, then remove media and wash with 1X PBS.
4. Add 500 µl of new media (DMEM which is free of phenol and serum) and incubate at at 37ºC for 18 hours. This is because the presence of lactate dehydrogenase may degrade lactate in the supernatant.
5. Use a pipette to remove all supernatant, transfer into a microcentrifuge tube, and centrifuge at 250 rcf for 3 min to remove cells that may have left in the supernatant.
6. Transfer the cell-free supernatant into fresh tubes.
7. Cell-free supernatant is used in GFP microscopy

Cell culture

Reagent and buffers

Sterile PBS

HyClone DMEM/ high glucose containing 10% Fetal Bovine Serum and 1X penicillin/streptomycin

TrypLE Express (Trypsin)

To make growth media:

HyClone DMEM/ high glucose media	450ml
Fetal Bovine Serum	50ml
100X Penicillin/Streptomycin	5ml

Thawing frozen stock of cells

* Warm two separate 15 ml falcon tubes containing 9 ml and 10 ml of cell media to 37^{o}C

* Label plates with cell line, passage number and date

1. Quickly thaw the frozen stock in a 37°C bath. Make sure the lid is tight and the cryo-tube remains upright in the bath to avoid contamination. As soon as ice crystals disappear, carefully wipe the sides of the tube with 70% ethanol to avoid contamination.
2. Transfer 1 ml of cells into 9 ml of pre-warmed media drop by drop
3. Spin down the cells at 125xg for 8 mins
4. Remove the supernatant without disturbing the cell pellet
5. Gently resuspend the cells in 10 ml of pre-warmed cell media by pipetting up and down

6. Transfer the suspension into a 10 cm cell culture dish
7. Place dish in 37 °C ± 1°C incubator 5% CO_2

Passaging cells

* Bring cell media and trypsin to 37^{o}C before starting the protocol

* Label plates with cell line, passage number and date

1. Check that cells are 70-80% confluent
2. Aspirate the media from the plate
3. Wash cells with 10ml of sterile PBS and aspirate off the PBS
4. Add 3 ml of Trypsin into the plate and leave it in the incubator for 2-3mins. If cells don't get detached, use more trypsin (< 1 ml) and incubate for ~1 – 2 more minutes or tap the flask.
5. Check under the microscope to ensure that all cells have detached from the plate
6. Add 7 ml of media into the plate
7. Pipette media up and down, and squirt it around the surface of the dish to remove adherent cells from the surface.
8. Add 8 ml of media into a new plate and add 2 ml of the trypsinised cells from the previous plate
9. Place in 37 °C ± 1°C incubator 5% CO_2

Making frozen stock of cells

To make 5 ml of freezing media,

Fetal Bovine Serum	4.5 ml
DMSO	0.5 ml

1. Spin down cells at 1000-1200 rpm for 5 mins.
2. Aspirate out the media and add freezing media
3. Put 1 ml of mixture per freezing vial
4. Put vials into CoolCell Cell freezing container overnight
5. Remove the vials and store at -80^{o}C the next day

Cell Lysis in NET with 0.5% TritonX100

Reagent and buffers

TritonX100

Sodium Chloride

0.5M EDTA

1M Tris pH 7.4

25X protease inhibitor

Sterile PBS

HyClone DMEM/ high glucose containing 10% Fetal Bovine Serum and 1X penicillin/streptomycin

TrypLE Express (Trypsin)

MiliQ water

To make lysis buffer:

1. Mix 20 mM Tris, 100 mM NaCl and 1 mM EDTA to create the NET buffer
2. Dilute stock Triton X100 solution to 10% in water then dilute to 0.5% in NET
3. Add 1X protease inhibitor

Cell lysis:

*All reactions should be done on ice

1. Add 3ml of trypsin to trypsinise cells for lysis and quench with 7ml of media
2. Spin cells down in 15 or 50 mL centrifuge tubes at 1500 rpm for 6 mins.
3. Aspirate off media and resuspend in 5ml of ice cold PBS
4. Spin cells down at 1500 rpm for 6 mins.
5. Resuspend cells in lysis buffer, with 20 μL of lysis buffer per million cells
6. Let cells sit in lysis buffer on ice for 10-15 minutes to ensure complete lysis.
7. Spin cells at the maximum speed of the microcentrifuge for 10 minutes to pellet nuclei.
8. Decant the supernatant into a new microcentrifuge tube and store at -20^{o}C

Immunostaining

Reagent and buffers

Cells of interest seeded in 35 mm dishes

1X PBS

16% Paraformaldehyde (PFA) (dilute 1 in 4 times in PBS to get 4% PFA)

Blocking buffer (0.5% Fetal Bovine Serum in PBS)

Primary antibody

Secondary antibody (eg Dylight Alexa-488 anti-mouse secondary antibodies)

MiliQ water

Nail polish

Protocol

1. One to two days before the immunostaining, seed cells in 35 mm dishes.
2. Remove media from dish
3. Wash with 1ml of PBS at room temperature
4. Remove PBS and add 500 ul of 4% PFA and incubate at room temperature for 10-15 mins
5. Remove PFA and wash twice with PBS
6. Add 1 ml of blocking buffer onto coverslips and incubate at room temperature for 15-20 min
7. Prepare the incubation chamber by wetting a few pieces of c-fold towels and placing an ice box to cover the towels to keep the environment moist
8. Place a piece of parafilm on the towels

9. Dilute the primary antibody in blocking buffer and spot 50ul of diluted primary antibody on the parafilm. Remove the coverslip with the cells from the dish and invert such that the cell side is in contact with the primary antibody solution
10. Incubate for 45 mins at room temperature
11. Wash the coverslips with PBS twice
12. Dilute the secondary antibody in blocking buffer and spot 50ul of diluted secondary antibody on the parafilm. Put the coverslip cell side down on the diluted secondary antibody
13. Incubate for 45 mins at room temperature
14. Wash the coverslips with PBS twice
15. Wash once in miliQ water
16. Mount coverslips on a glass slide with mounting media
17. Use nail polish to line the edges of the coverslip
18. Incubate the coverslip for 20 mins in the oven or overnight at room temperature
19. View the slide on a fluorescence microscope

Western Blot

Gel Casting (10% resolving gel and a 4% stacking gel)

1. Prepare the gel according to Table 1 and add APS and TEMED only when ready to pour the gel into the plates.

Table 1: Composition of 10% resolving gel (for 2 mini gels)

Composition	Stock concentrations	Final concentration	Volume to add
H_2O	N.A.	N.A.	4.106 ml
1.5M Tris-HCl, 0.4% SDS, pH8.8	1.5M, 0.4% SDS	0.375M, 0.1% SDS	2.5 ml
Acrylamide	30%	10%	3.334 ml
APS	10%	0.05%	50 µl
TEMED (Add last)	N.A.	N.A.	10 µl
Total			**10 ml**

2. Apply the gel contents into the glass plate sandwich with a dropper until the level 1cm below wells.

3. Overlay quickly with ethanol to the top of the short plate.

4. Wait until the polyacrylamide has polymerized (about 30 minutes) before continuing with the stacking gel. You will see a distinct line between the polyacrylamide gel and ethanol when the gel has completely polymerized.

5. After polymerization, prepare the stacking gel (according to Table 2) and pour away the ethanol
from the top of the resolving gel just before overlaying the resolving gel with the stacking gel.

Table 2: Composition of 4% stacking gel (for 2 mini gels)

Composition	Stock concentrations	Final concentration	Volume to add
H_2O	N.A.	N.A.	3.028 ml
0.5M Tris-HCl, 0.4% SDS, pH 6.8	0.5M, 0.4% SDS	0.125M, 0.1% SDS	1.25 ml
Acrylamide	30%	4.0%	0.667ml
APS	10%	0.1%	50 µl
TEMED (Add last)	N.A.	N.A.	5 µl
Total			**5 ml**

6. Carefully insert the comb before the gel solidifies and ensure that no bubbles are trapped below
the wells. Allow the gel to solidify for at least 30 minutes. While waiting, you can prepare the samples now.

7. After the gel has solidified, remove the comb and assemble it into the tank. Fill the tank with SDS gel running buffer.

SDS PAGE

1. Prepare protein samples with 6X SDS PAGE loading dye (as CD44v6 is a membrane protein, samples were not heated for denaturation)

2. Pipette 5 µl standard marker and all of the 24 µl of the sample and load it into the respective wells. Ensure there is no spillage of samples into adjacent wells.

Starting the electrophoresis

1. Check to make sure that there is no leakage of buffer in the inner chamber.

2. Set the power pack to run at 70 V for 30 mins before running at 100 V for one hour
3. After the electrophoresis has completed its run, pry open the gel sandwich using a wedge

4. Remove the short plate while ensuring the gel remains on the spacer plate.

5. Using the wedge again, slice the gel longitudinally into 2 pieces to separate the duplicate samples and mark the orientation of the gel. You should cut between Lane 5 and Lane 6 carefully.

6. Use half of the gel for Coomasie blue staining and the other half for Western blot.

Coomasie Blue staining

1. Soak the gel in 20 ml of InstantBlue staining solution for 10 – 20 minutes.

2. Discard the staining solution into a reagent waste bottle.

3. Add destaining solution and shake the gel for 15 mins.

4. Pour away the destaining solution and add fresh destaining solution. Continue this process until bands are clear enough for photo taking.

Semi-dry transfer for Western Blot

1. Prepare 2 pieces of fibre pads, one Polyvinylidene fluoride (PVDF) membrane and the semi-dry transfer apparatus

2. Pre-wet the PVDF membrane in 100% methanol until the membrane turns translucent. Methanol is toxic and should not be handled with bare hands.

3. Prepare the gel sandwich as follows:
- Place fibre pad on the cathode
- Place membrane on fibre pad
- Place gel on membrane
- Complete the sandwich by placing the second piece of fibre pad on the gel

*Add transfer buffer after every layer has been added to ensure that the sandwich is sufficiently wet
* Use a glass tube or roller to gently roll out air bubbles.

4. Close the apparatus tightly, plug the cables into the power supply, and run the blot. Set the power pack to run at a constant voltage of 15 V for 1 hr.

Immunodetection

1. Block the nonspecific sites on the blot in 10 ml of 5% blocking buffer (0.5 g of low fat milk in 10 ml of TBS-T) for 30 minutes at room temperature with shaking.

2. Wash the blot in 15 ml of TBS-T for 5 minutes at room temperature with shaking.

3. Add 5 ml of the primary antibody (anti-CD44v6 VFF18 diluted 1:500 with TBS-T) and incubate overnight at 4oC with shaking.

4. Wash the blot 3 times for 15 minutes with 15 ml of TBS-T.

5. Add 5 ml of secondary antibody (anti-mouse IgG diluted 1:10000 with TBS-T) and incubate for 1 hour at room temperature with shaking.

6. Wash the blot 3 times for 10 minutes with 15 ml of TBS-T.

7. Pour away the TBS-T and add 1 ml each of the home-made substrates

8. Visualise the blot using a chemiluminescence imager

Molecular Cloning Protocols

PCR Protocol

In the polymerase chain reaction (PCR) experiments conducted by the NUS_Singapore team, two enzymes are mainly used: *Taq* polymerase and *Q5* polymerase. The reagents and proportions used are outlined below.

1 Taq PCR

1.1 Reagents and amounts used

Table 1.1: Reagents and the corresponding proportions of each reagent for a 20 and 50 µl *Taq* PCR reaction.

Reagents	25 µl reaction (µl)	50 µl reaction (µl)
10X Standard *Taq* Reaction buffer	2.5	5
10 mM dNTP	0.5	1
$MgCl_2$	2	4
10 µM forward primer	0.5	1
10 µM reverse primer	0.5	1
Template DNA	Variable	Variable
Taq DNA polymerase	0.5	1
Nuclease-free water	to 25	to 50

Protocol adapted from: https://www.neb.com/protocols/1/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273

1. Mix the reagents together on ice in the proportions shown in Table 1.1 and mix by tapping (do not vortex).
2. Do a short spin on the centrifuge to bring down all of the mixture.
3. Place the tubes into the PCR machine and set the cycling conditions as shown in Table 1.2.

1.2 Cycling conditions

Table 1.2: Cycling steps, temperature and the corresponding time and number of cycles used for the *Taq* PCR reaction.

Cycling steps	Temperature (^{o}C)	Time (s)	Number of cycles
Initial denaturation	95	30	1
Denaturation	95	10 to 30	30 to 35
Annealing	55 to 72	30	
Extension	72	60 per kb	
Final extension	72	300	1
Hold	4	Infinite	-

Protocol adapted from: https://www.neb.com/protocols/1/01/01/taq-dna-polymerase-with-standard-taq-buffer-m0273

2 Q5 PCR

2.1 Reagents and amounts used

Table 2.1: Reagents and the corresponding proportions of each reagent for a 20 and 50 µl *Q5* PCR reaction.

Reagents	25 µl reaction (µl)	50 µl reaction (µl)
5X Q5 reaction buffer	5	10
10 mM dNTP	0.5	1
10 µM forward primer	1.25	2.5
10 µM reverse primer	1.25	2.5
Template DNA	Variable	Variable
Q5 high-fidelity DNA polymerase	0.5	1
Nuclease-free water	to 25	to 50

Protocol adapted from: https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491

1. Mix the reagents together on ice in the proportions shown in Table 2.1 and mix by tapping (do not vortex).
2. Do a short spin on the centrifuge to bring down all of the mixture.

3. Place the tubes into the PCR machine and set the cycling conditions as shown in Table 2.2.

2.2 Cycling conditions

Table 2.2: Cycling steps, temperature and the corresponding time and number of cycles used for the *Q5* PCR reaction.

Cycling steps	Temperature (°C)	Time (s)	Number of cycles
Initial denaturation	98	30	1
Denaturation	98	10	30 to 35
Annealing	55 to 72	30	
Extension	72	30 per kb	
Final extension	72	300	1
Hold	4	Infinite	-

Protocol adapted from: https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491

Restriction Enzyme Digest

The optimal buffer to be used for the reaction was found using the NEB Double Digest Finder (http://www.neb.sg/tools-and-resources/interactive-tools/double-digest-finder).

3 Reagents and amount used

Table 1: Reagents and the corresponding amount required for a 50 µl restriction digest reaction.

Reagents	For a 50 µl reaction
DNA	Variable (usually around 1 µg)
10X buffer (either NEBuffer 3.1 or CutSmart Buffer)	5 µl
Restriction Enzyme	1 µl
Nuclease-free water	To 50 µl

Protocol adapted from: http://nebcloner.neb.com/#!/protocol/re/single/EcoRI

1. Prepare the mixture as shown in Table 1.
2. Gently flick the tube to mix the mixture and do a short spin using a centrifuge to bring down all of the solution.
3. Place the tube in a heat block previously set at 37ºC for around 1 to 3 hours.
4. If the reaction is not immediately purified or ran on gel, perform heat inactivation at 65ºC or 80ºC depending on the enzyme used (see https://www.neb.com/tools-and-resources/usage-guidelines/heat-inactivation).

Ligation

Ligation was done at a 3:1 insert to vector ratio according to the NEB calculator http://nebiocalculator.neb.com/#!/ligation.

4 Reagents and amount used

Table 2: Reagents and the corresponding amount required for a 20 µl ligation reaction.

Reagents	For a 20 µl reaction
10X T4 DNA Ligase Buffer	2 µl
Vector DNA	Variable
Insert DNA	Variable
Nuclease-free water	To 20 µl
T4 DNA Ligase*	1 µl

Protocol adapted from: https://www.neb.com/protocols/1/01/01/dna-ligation-with-t4-dna-ligase-m0202

**Note that T4 DNA Ligase has to be added last*

1. Prepare the mixture as shown in Table 2. Note that T4 DNA Ligase should be added last.
2. Gently flick the tube to mix the mixture and do a short spin using a centrifuge to bring down all of the solution.
3. Leave the tube on bench overnight (approximately 16 hours).